cDNA Cloning, Heterologous Expressions, and Functional Characterization of Malonyl-Coenzyme A:Anthocyanidin 3-*O*-Glucoside-6″-*O*-Malonyltransferase from Dahlia Flowers[1]

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In the flowers of important ornamental Compositae plants, anthocyanins generally carry malonyl group(s) at their 3-glucosyl moiety. In this study, for the first time to our knowledge, we have identified a cDNA coding for this 3-glucoside-specific malonyltransferase for anthocyanins, i.e. malonyl-coenzyme A:anthocyanidin 3-*O*-glucoside-6″-*O*-malonyltransferase, from dahlia (*Dahlia variabilis*) flowers. We isolated a full-length cDNA (*Dv3MaT*) on the basis of amino acid sequences specifically conserved among anthocyanin acyltransferases of the versatile plant acyltransferase family. *Dv3MaT* coded for a protein of 460 amino acids. Quantitative real-time PCR analyses of *Dv3MaT* showed that the transcript was present in accordance with the distribution of 3MaT activities and the anthocyanin accumulation pattern in the dahlia plant. The *Dv3MaT* cDNA was expressed in *Escherichia coli*, and the recombinant enzyme was purified to homogeneity and characterized. The recombinant Dv3MaT catalyzed the regiospecific transfer of the malonyl group from malonyl-coenzyme A (K_m, 18.8 μM) to pelargonidin 3-*O*-glucoside (K_m, 46.7 μM) to produce pelargonidin 3-*O*-6″-*O*-malonylglucoside with a k_{cat} value of 7.3 s^{-1}. The other enzymatic profiles of the recombinant Dv3MaT were closely related to those of native anthocyanin malonyltransferase activity in the extracts of dahlia flowers. *Dv3MaT* cDNA was introduced into petunia (*Petunia hybrida*) plants whose red floral color is exclusively provided by cyanidin 3-*O*-glucoside and 3,5-*O*-diglucoside. Thirteen transgenic lines of petunia were found to produce malonylated products of these anthocyanins (11–63 mol % of total anthocyanins in the flower). The spectral stability of cyanidin 3-*O*-6″-*O*-malonylglucoside at the pHs of intracellular milieus of flowers was significantly higher than that of cyanidin 3-*O*-glucoside. Moreover, 6″-*O*-malonylation of cyanidin 3-*O*-glucoside effectively prevented the anthocyanin from attack of β-glucosidase. These results suggest that malonylation should serve as a strategy for pigment stabilization in the flowers.

Anthocyanins, the largest subclass of plant flavonoids, are in many cases responsible for the orange, red, purple, and blue colors in flowers and exist as glycosylated, acylated, and/or methylated forms in vacuoles (Strack and Wray, 1994). Acyl substituents of anthocyanins are commonly linked to a hydroxy group of a glycosyl moiety of anthocyanins. The enzymatic acylation of anthocyanins generally occurs under strict regiocontrol during the late stage of their biosynthesis. It has been proposed that aromatic acylation makes anthocyanins more stable and bluer by intramolecular stacking of the anthocyanins with polyphenols (Goto and Kondo, 1991; Brouillard and Dangles, 1994), whereas aliphatic acylation of anthocyanin, such as malonylation, is believed to be important for enhancing the pigment solubility in water, protecting glycosides from enzymatic degradation, stabilizing anthocyanin structures, or uptaking anthocyanins into vacuoles (Heller and Forkmann, 1994).

The malonyl group is one of the most common aliphatic acyl substituents of anthocyanins. In the commercially important ornamental flowers of Compositae plants, such as dahlia (*Dahlia variabilis*; Yamaguchi et al., 1999), cineraria (Goto et al., 1984), and chrysanthemum (Nakayama et al., 1997), the malonyl group(s) is linked to the 3-glucosyl moiety of anthocyanins. So far, the occurrence of an enzyme activity specifically catalyzing the malonyl-CoA-dependent malonylation of the 3-glucosyl moiety of anthocyanins (i.e. malonyl-CoA:anthocyanidin 3-*O*-glucoside-6″-*O*-malonyltransferase; termed 3MaT, Fig. 1A) has been demonstrated in the crude extracts

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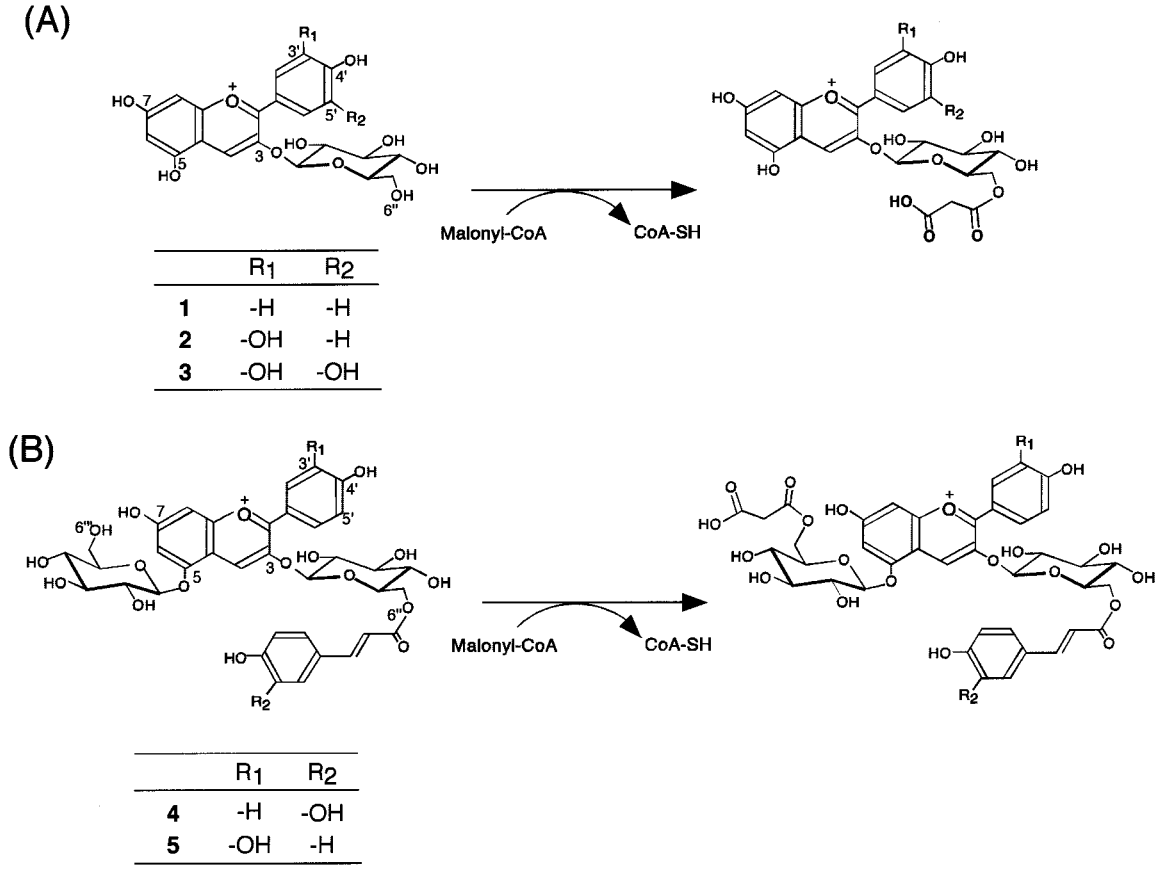

	R_1	R_2
1	-H	-H
2	-OH	-H
3	-OH	-OH

	R_1	R_2
4	-H	-OH
5	-OH	-H

Figure 1. Regiospecific malonyl transfer catalyzed by 3MaT from dahlia (A) and Ss5MaT1 from *S. splendens* (B). Key positional numberings of anthocyanins are labeled on substrates. Substrate names are: 1, pelargonidin 3-*O*-glucoside; 2, cyanidin 3-*O*-glucoside; 3, delphinidin 3-*O*-glucoside; 4, bisdemalonylsalvianin; and 5, shisonin. For the sake of convenience, structures of anthocyanins are shown as their flavylium forms.

of dahlia flowers (Yamaguchi et al., 1999), and a biosynthetic pathway leading to their major anthocyanins has also been proposed (Ogata et al., 2001; Yamaguchi et al., 1999). However, a gene coding for this anthocyanin malonyltransferase remains to be identified.

A cDNA coding for the malonyl-CoA:anthocyanin 5-*O*-glucoside-6‴-*O*-malonyltransferase of *Salvia splendens* (Ss5MaT1; Suzuki et al., 2001), which catalyzes the regiospecific transfer of the malonyl group to the 6‴-*O*-hydroxyl group of the 5-*O*-glucoside moiety of anthocyanin 5-*O*-glucosides, has recently been isolated from *S. splendens* flowers (Fig. 1B). Sequence comparison studies suggest that this anthocyanin malonyltransferase belongs to a versatile plant acyltransferase family. This protein family consists of acyl-CoA-dependent acyltransferases that play important roles in secondary metabolism in plants, such as those of anthocyanins (Fujiwara et al., 1998; Yonekura-Sakakibara et al., 2000), phytoalexins (Yang et al., 1997), vindoline (St-Pierre et al., 1998), benzylacetate (Dudareva et al., 1998), taxol (Walker and Croteau, 2000a, 2000b; Walker et al., 2000), and morphine (Grothe et al., 2001). However, biochemically characterized members of this family should represent only a small fraction of the total membership of the family because it was recently estimated that Arabidopsis contains about 70 related genes of this family (Dudareva and Pichersky, 2000). Although two highly conserved sequences (motif 1 [Fig. 2] and motif 3) have been ubiquitously identified among members of the family, the entire amino acid sequences of the family members show only low similarities with each other. For example, despite the similarity in the biochemical role of anthocyanin acyltransferases of this family, they show only 30% to 40% sequence identity with each other. We also recently identified a signature sequence called motif 2, which is specifically conserved among anthocyanin acyltransferases of the family.

In this paper, the first (to our knowledge) identification of a cDNA coding for 3MaT from dahlia flowers is described. The putative anthocyanin acyltransferase cDNA could be specifically isolated by a homology-based strategy taking full advantage of the specific conservation of motif 2 in anthocyanin acyltransferases. Obtaining a 3MaT sequence allowed us to execute the first sequence comparison studies among anthocyanin acyltransferases of different

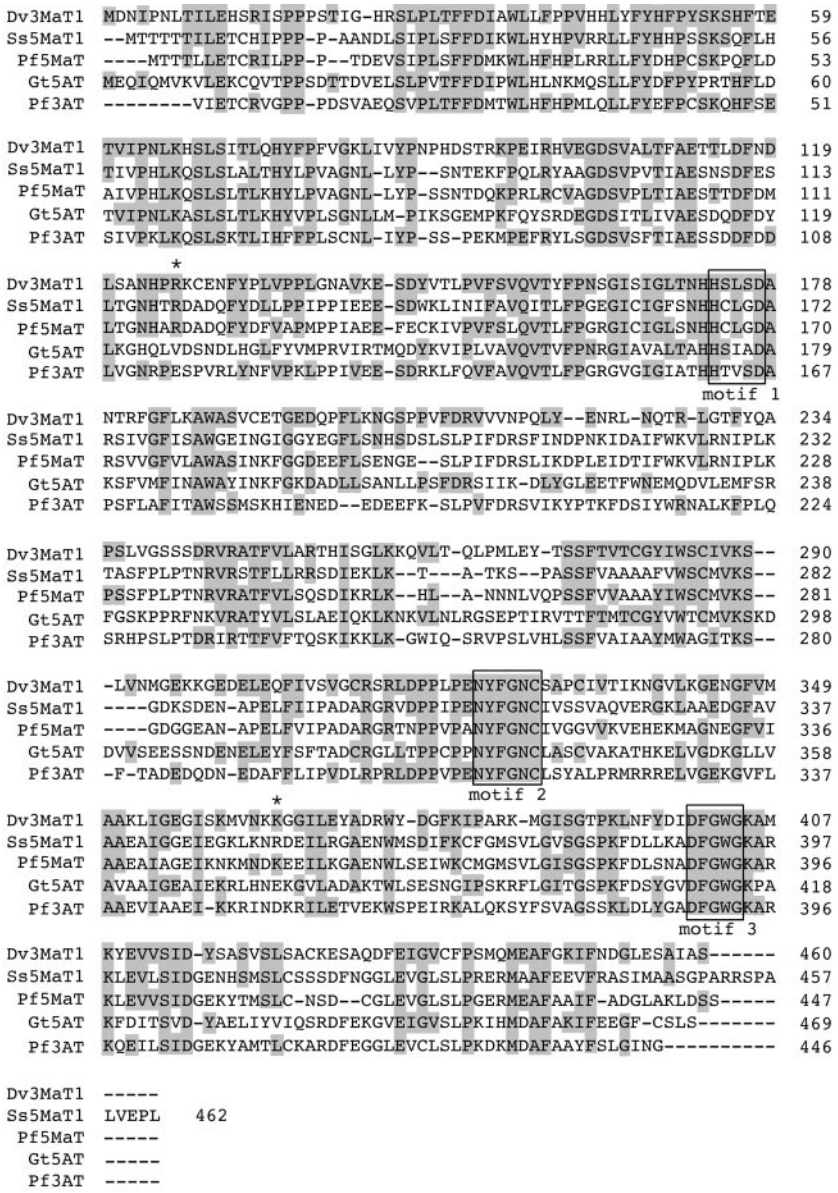

Figure 2. Alignment of the deduced amino acid sequence of Dv3MaT with those of anthocyanin acyltransferases of different specificities. Amino acid residues identical to that of Dv3MaT are shadowed. Motifs 1 through 3 are boxed. Cationic amino acid residues that are specifically conserved among malonyltransferases are indicated by asterisks above the Dv3MaT sequence. The enzymes used for alignment are Ss5MaT1 (malonyl-CoA:anthocyanin 5-*O*-glucoside-6‴-*O*-malonyltransferase from *S. splendens*; GenBank accession no. AF405707), Pf5MaT (malonyl-CoA: anthocyanin 5-*O*-glucoside-6‴-*O*-malonyltransferase from *P. frutescens*; GenBank accession no. AF405204), Gt5AT (hydroxycinnamoyl-CoA: anthocyanin 5-*O*-glucoside-6‴-*O*-acyltransferase of *G. triflora*; GenBank accession no. BAA74428), and Pf3AT (hydroxycinnamoyl-CoA:anthocyanin 3-*O*-glucoside-6″-*O*-acyltransferase of *P. frutescens*; GenBank accession no. BAA93475).

specificities to explore putative amino acid residues involved in the specificity determination. In addition, transgenic petunia (*Petunia hybrida*) plants harboring this cDNA have been created and analyzed for the production of malonylated anthocyanins in the plant, providing the first example, to our knowledge, of the functional expression of the malonyltransferase gene in a heterologous plant system. The functional significance of anthocyanin malonylation is discussed on the basis of these results, along with stability studies of malonylated and non-malonylated forms of the pigments.

RESULTS

cDNA Cloning

A partial cDNA of 250 bp, termed *AT927*, was obtained by PCR using a cDNA library prepared from red petals of dahlia cv Kaen and primers designed on the basis of the amino acid sequences of motifs 2 and 3 of acyltransferases (see "Discussion"; Fig. 2; Suzuki et al., 2001). Using the *AT927* fragment as a probe, the cDNA library of 40,000 clones was screened under high-stringency conditions, giving rise to 14 positive clones. The nucleotide sequences of clones with the longest insert were determined. The full-length cDNA, termed *Dv3MaT* (GenBank accession no. AF489108), coded for a protein of 460 amino acids with a calculated M_r of 51,301 (Fig. 2). The deduced amino acid sequence of Dv3MaT showed the highest sequence similarity to anthocyanin malonyltransferases from *S. splendens* (Ss5MaT1, 39% identity; Suzuki et al., 2001) and *Perilla frutescens* (Pf5MaT, 38%; GenBank accession no. AF405204), hydroxycinnamoyl-CoA:anthocyanin 5-glucoside-6‴-*O*-acyltransferase of *Gentiana tri-*

flora (Gt5AT, 36%; Fujiwara et al., 1998), and hydroxycinnamoyl-CoA:anthocyanin 3-glucoside-6″-*O*-acyltransferase *P. frutescens* (Pf3AT, 34%; Yonekura-Sakakibara et al., 2000; Fig. 2). Phylogenetic analysis showed that Dv3MaT is a member of the anthocyanin acyltransferase subfamily (Suzuki et al., 2001) of the plant versatile acyltransferase family; it is phylogenetically distant from other members of the family, such as those involved in the biosyntheses of phytoalexins (Yang et al., 1997), vindoline (St-Pierre et al., 1998), benzylacetate (Dudareva et al., 1998), taxol (Walker and Croteau, 2000a, 2000b; Walker et al., 2000), and morphine (Grothe et al., 2001).

Quantitative Real-Time PCR

To evaluate the functional significance of *Dv3MaT* in the anthocyanin biosynthesis in dahlia flowers, its spatial and temporal expressions in the dahlia plant were analyzed by quantitative real-time PCR, and the results were compared with the distribution of 3MaT activity and pigmentation patterns of the dahlia plant. The real-time PCR was executed using first-strand cDNA libraries of respective organs as a template and primers specific for *Dv3MaT* (see Fig. 3). The *Dv3MaT* transcript was abundant in red petals and the red stem, and the highest level of transcription was observed in petals at stage 2 of flower development; on the other hand, little or no transcript could be found in leaf, bract, sepals, and white petals. These observations were in good agreement with the distribution of 3MaT activity and pigmentation patterns in the dahlia plants (Fig. 3).

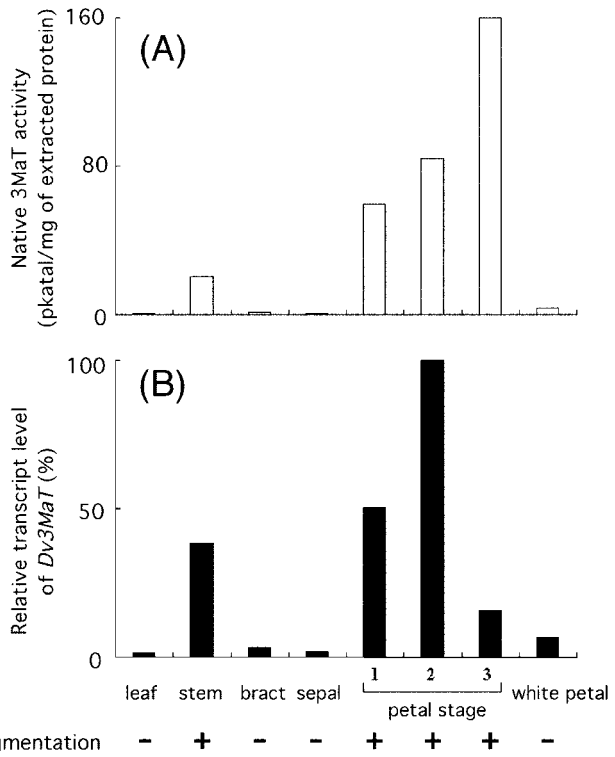

Figure 3. Relationship between the activity of native 3MaT (A, white bars) and the relative transcript level of *Dv3MaT* (B, black bars) in individual organs of a dahlia cv Kaen plant, i.e. leaves (green), stems (red), bracts, sepals, and petals of three developmental stages (stage 1, closed bud, 4–6 mm in length, half-pigmented; stage 2, top of the bud is open, 6–9 mm in length, almost pigmented; and stage 3, fully open flower, 18–22 mm in length, fully pigmented). White petals (15–20 mm in length), taken from a fully open flower of the acyanic cultivar of dahlia, were also analyzed for the transcription of *Dv3MaT*. Transcription of *Dv3MaT* was detected by quantitative real-time PCR using first-strand cDNA as a template, synthesized on 20 ng of total RNA. SEs of determination of *Dv3MaT* transcripts were within ±30%. The plus and minus signs indicate the presence and absence of red pigmentation in individual organs of dahlia, respectively.

Dv3MaT: Expression in *Escherichia coli*, Characterization, and Specificity

To compare the enzymatic properties of Dv3MaT with those of native 3MaT, the *Dv3MaT* cDNA was expressed in *E. coli* cells, and the recombinant enzyme was purified to homogeneity and characterized. The recombinant Dv3MaT was a soluble, catalytically active form of protein. The recombinant Dv3MaT could be purified to homogeneity from crude extracts of transformant cells by three chromatographic steps with an activity yield of 28% (see "Materials and Methods"). The purified Dv3MaT showed a strong 3MaT activity: It could catalyze the malonyl-CoA-dependent malonyl transfer to pelargonidin 3-*O*-glucoside to produce pelargonidin 3-*O*-6″-*O*-malonylglucoside (Fig. 1A), the structure of which was confirmed by 500-MHz ^{1}H-NMR as well as mass spectrometry (MS) analyses (data not shown). The kinetic parameters for the malonylation of pelargonidin 3-*O*-glucoside were as follows: k_{cat}, 7.3 s^{-1}; K_m for malonyl-CoA, 18.8 μM; and K_m for pelargonidin 3-*O*-glucoside, 46.7 μM. We also examined the specificities of Dv3MaT toward other acyl acceptors and donors, and the results are summarized in Table I.

The enzyme was active over a pH range of 5.5 to 11.0, with a maximum activity at pH 8.5, and was stable at 6.0 to 7.5 (at 20°C for 20 h) and below 45°C (at pH 7.0 for 20 min). The recombinant Dv3MaT was completely inhibited by 5 mM *N*-ethylmaleimide or 0.1 mM Cu^{2+} after incubation at 20°C for 20 min and was partially inhibited by 0.1 mM Fe^{2+} (residual activity, 36%), 0.1 mM Hg^{2+} (35%), and 0.1 mM acetyl-CoA (87%). Other metal ions (Ca^{2+}, Cd^{2+}, Co^{2+}, Mg^{2+}, Mn^{2+}, Ni^{2+}, Sn^{2+}, and Zn^{2+}; 0.1 mM) and 5 mM EDTA had negligible effects on enzyme activity.

Expression of Dv3MaT in Petunia

We created a transgenic petunia harboring *Dv3MaT* cDNA (Fig. 4B) using a petunia plant as a

Table I. *Substrate specificity of recombinant Dv3MaT*

nd, Not determined.

	Relative Activity[a]	k_{cat}[b]	K_m[b]	k_{cat}/K_m
	%	s^{-1}	mM	s^{-1} mM^{-1}
Acyl donor[c]				
Malonyl-CoA	100	7.3	18.8	0.39
Acetyl-CoA	<0.1	nd		
Methylmalonyl-CoA	11	5.5	534	0.01
Succinyl-CoA	16	0.7	22.5	0.03
Caffeoyl-CoA	<0.1	nd		
Acyl acceptor[d]				
Pelargonidin 3-*O*-glucoside[e]	100	7.3	46.7	0.16
Cyanidin 3-*O*-glucoside[e]	111	7.8	39.9	0.20
Delphinidin 3-*O*-glucoside[e]	117	9.0	45.6	0.20
Quercetin 3-*O*-glucoside[f]	15	1.3	80.9	0.02
Cyanidin 3-*O*-6″-*O*-malonylglucoside[f]	0.25	nd		
Pelargonidin 3,5-*O*-diglucoside[f]	1.9	nd		
Pelargonidin 3-*O*-6″-*O*-malonylglucoside-5-*O*-glucoside[f]	<0.1	nd		
Shisonin[g]	4.2	nd		

[a]For the relative activities of acyl donors (final concentration, 60 μM) and acyl acceptors (final concentration, 120 μM), the specific activity with malonyl-CoA and pelargonidin 3-*O*-glucoside (135 nkat mg^{-1}) was taken to be 100%. Assay conditions are described under "Materials and Methods." The relative activities were determined from product peak integrals, assuming that the extinction coefficient of the reaction product was the same as that of the substrate. [b]The SEs of kinetic parameters were within ±20%. [c]The reactions were performed with pelargonidin 3-*O*-glucoside as an acyl acceptor. [d]The reactions were performed with malonyl-CoA as an acyl donor. [e]The structures of 3-*O*-glucosides of pelargonidin, cyanidin, and delphinidin are presented in Figure 1A. [f]We did not determine the site of malonylation in the reaction product. [g]The structure of shisonin is presented in Figure 1B. In the analytical HPLC, the reaction product was co-eluted with malonylshisonin, which is a product of the Ss5MaT1-catalyzed malonyl transfer to shisonin.

host whose red flower color was provided by cyanidin 3-*O*-glucoside and 3,5-*O*-diglucoside. Nineteen independent transgenic petunia plants (T1) were obtained. RT-PCR analysis detected the transcript derived from *Dv3MaT* in 13 plants. HPLC-MS analysis of anthocyanins in the flowers of these transgenic lines (Fig. 4A for example) revealed that these lines contained cyanidin 3,5-*O*-diglucoside (peak *a*; λ_{max}, 515 nm) and cyanidin 3-*O*-glucoside (peak *b*; λ_{max}, 519 nm), along with anthocyanins that were not found in the host (peak *c*; λ_{max}, 515 nm; and peak *d*; λ_{max}, 519 nm). The molecular ions of the peaks *c* and *d* were shown to be m/z 697 $[M]^+$ and m/z 535 $[M]^+$ by on-line mass spectrometric analysis, strongly suggesting that these peaks were those of cyanidin 3-*O*-6″-*O*-malonylglucoside-5-*O*-glucoside and cyanidin 3-*O*-6″-*O*-malonylglucoside, respectively. The molar ratio of the malonylated anthocyanins out of total anthocyanins in the flower was estimated from absorbance-peak integrals at 520 nm to be 11.0 to 62.8 mol %, depending on the transgenic lines. The flower color of these transgenic lines did not significantly differ from that of the host. This is not surprising because malonylation of anthocyanins did not change their absorption maxima.

Evaluation of the Effect of 6″-*O*-Malonylation on the Stability of Anthocyans

We examined the effect of 6″-*O*-malonylation of anthocyanin on the pigment stability in in vitro aqueous systems. In such systems, anthocyanins do not exist in their stable colored forms at pH 4 (vacuolar pHs) to 7 (cytosolic pH) but rapidly undergo decoloration, which is attributable to hydration of the pigments to produce a colorless carbinol pseudobase species (Brouillard and Dangles, 1994). In in vitro aqueous systems, anthocyanin is proposed to exist as several distinct forms, depending on pH, as follows (Brouillard and Dangles, 1994). At acidic pH (<2), anthocyanin is present in its cationic flavylium form, which is red in color. At pH 4 to 7, the neutral 7- and 4-quinonoidal base isomers (bluish-purple in color) are produced from the flavylium cation through prototropic tautomerism, which subsequently results in the production of the anionic 7- and 4-quinonoidal base isomers (blue in color) at pH higher than 7. At pH higher than 5, the anthocyans also undergo hydration to produce a colorless carbinol pseudobase species. Thus, in the absence of any stabilizing effect, anthocyanins are expected to remain poorly colored in the flower sap. It has been suggested that the

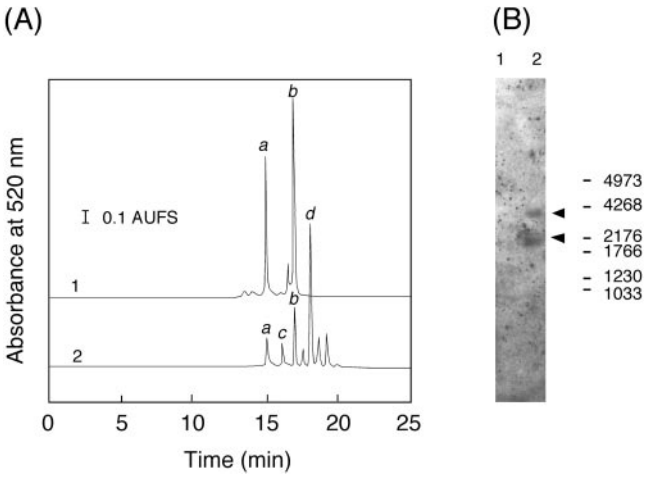

Figure 4. A, HPLC analysis of anthocyanin compositions of flowers from a non-transgenic line (chromatogram 1) and a transgenic line harboring *Dv3MaT* cDNA (chromatogram 2) of petunia as monitored by 520 nm. Peaks a, b, c, and d are those of cyanidin 3,5-*O*-diglucoside, cyanidin 3-*O*-glucoside, cyanidin 3-*O*-6″-*O*-malonylglucoside-5-glucoside, and cyanidin 3-*O*-6″-*O*-malonylglucoside, respectively, as determined by on-line mass spectrometric analysis. B, Southern-blot analysis of *Hin*dIII-digested genomic DNA of the corresponding non-transgenic (lane 1) and transgenic (lane 2) lines of petunia using *Dv3MaT* cDNA as a probe. Arrowheads indicate positive signals, suggesting the existence of two copies of this cDNA in the genome of this transgenic line. The scale indicates molecular sizes (in base pairs). For details, see "Materials and Methods."

malonylation of anthocyanin enhances pigment stability in aqueous systems (Saito et al., 1988). To further evaluate this observation and find any functional significance of anthocyanin malonylation, we compared the in vitro stability of the color intensities (i.e. A_{520} [at pHs 2.0 and 5.0] and A_{550} [at pH 7.0]) of the malonylated and non-malonylated forms of anthocyans: cyanidin 3-*O*-glucoside (a substrate for Dv3MaT), cyanidin 3-*O*-6″-*O*-malonylglucoside (the malonylation product), and cyanidin (their aglycon; Table II). At pH 2.0, the color intensities of these anthocyans, which probably exist as their flavylium forms, were essentially unchanged for several hours (not shown). However, at pH 7.0 (cytosolic pH) and 5.0 (putative pH of vacuoles), the color intensity of these anthocyans decreased with time and then became essentially constant after incubation for a prolonged period of time. The percentages of initial color intensity after incubation under these conditions for 1 h (at pH 5.0) or 24 h (at pH 7.0) are shown in Table II. The values greatly depended on the degree of anthocyan modification and were the highest with cyanidin 3-*O*-6″-*O*-malonylglucoside, followed by cyanidin 3-*O*-glucoside and cyanidin, in this order, at both pHs. For comparison, the color intensity of petal extract supplemented with cyanidin 3-*O*-6″-*O*-malonylglucoside (pH 5.1; for details, see "Materials and Methods") was unchanged (100%) after incubation at 30°C for 1 h. The decoloration processes of cyanidin 3-*O*-6″-*O*-malonylglucoside and cyanidin 3-*O*-glucoside could be fully reversed by acidification, which resulted in the transformation of their colorless forms into colored flavylium forms (Table II). However, the colorless form(s) of cyanidin could only partially (13%–23%) revert to its flavylium form upon acidification. Thus, the reversibility of the decoloration process also depended on the degree of modification of anthocyans. We also examined the effect of 6″-*O*-malonylation on the susceptibility of cyanidin 3-*O*-glucoside to the enzymatic degradation by β-glucosidase (cellulase; Table II). The results showed that cyanidin 3-*O*-glucoside was almost completely degraded by the enzyme, whereas 95% of

Table II. *Effect of 6″-O-malonylation on the stability of anthocyans*

Under "Pigment stabilities," an anthocyan pigment (final concentration, 50 μM) was incubated at 30°C in a 20 mM sodium acetate buffer (pH 5.0) for 1 h or a 20 mM potassium phosphate buffer (pH 7.0) for 24 h in a cuvette. A_{520} (at pH 5.0) or A_{550} (pH 7.0) was measured before and after the incubation using a double-beam spectrophotometer (model U-2000, Hitachi, Tokyo), and the percentages of initial absorbance after the incubation were determined. Aliquots of the 1-h incubation mixtures were also analyzed by HPLC (system A, see "Materials and Methods" for conditions) for the amount of anthocyan, which could revert from its colorless form(s) to the colored flavylium form upon acidification to pH <2 resulting from the HPLC conditions. The recovery (%) of the flavylium form of anthocyanin is shown in parentheses. Under "Resistance to β-glucosidase action," an anthocyan pigment (final concentration, 50 μM) was incubated at 30°C for 1 h in a 20 mM sodium acetate buffer (pH 5.0) containing 5 mg mL^{-1} β-glucosidase (cellulase "Onozuka"; Yakult, Tokyo). After incubation, aliquots of the incubation mixtures were analyzed by HPLC (system A) for the percentage of remaining anthocyans after the 1-h incubation.

Anthocyan	Remaining Percentage			
	Pigment stabilities			Resistance to β-glucosidase action, pH 5.0
	Buffer		Petal extract, pH 5.1	
	pH 5.0	pH 7.0		
Cyanidin	0 (23)	10 (13)	0 (17)	
Cyanidin 3-*O*-glucoside	4 (100)	30 (100)	31 (100)	2.6
Cyanidin 3-*O*-6″-*O*-malonylglucoside	52 (100)	62 (100)	100 (100)	95

cyanidin 3-*O*-6″-*O*-malonylglucoside remained undegraded after incubation for 1 h under the same conditions.

DISCUSSION

Homology-Based Strategy for the Cloning of Anthocyanin Acyltransferase cDNA

In an attempt to isolate anthocyanin acyltransferase cDNA by PCR through a homology-based strategy, it had been suggested that specific amplification of anthocyanin malonyltransferase genes based on the sequences of motifs 1 and 3 was rather difficult (H. Suzuki, T. Nakayama, and T. Nishino, unpublished data), probably because a single plant species should have numerous homologs of the family, corresponding to the versatility of the family members in the plant secondary metabolism (Dudareva and Pichersky, 2000). Thus, our present strategy took full advantage of the specific conservation of motif 2 among anthocyanin acyltransferases and was based on the sequences of motifs 2 and 3. Finally, we could obtain *Dv3MaT* cDNA that code for a putative anthocyanin acyltransferase belonging to the versatile acyltransferase family.

Evidence That *Dv3MaT* Is Responsible for the Biosynthesis of Malonylated Anthocyanins in Dahlia Flowers

The analysis of the temporal and spatial expressions of *Dv3MaT* in the dahlia plant showed that this cDNA was expressed in accordance with the distribution of native 3MaT activity and the pigment accumulation pattern. The recombinant Dv3MaT effectively catalyzed the malonyl-CoA-dependent malonyl transfer to pelargonidin 3-*O*-glucoside to produce pelargonidin 3-*O*-6″-*O*-malonylglucoside with a k_{cat} value of 7.3 s^{-1}, which was comparable with the value (7.2 s^{-1}) reported for the anthocyanin 5-glucoside-specific malonyltransferase, Ss5MaT1, of *S. splendens* (Suzuki et al., 2001). The highest acyl-donor preference of Dv3MaT for malonyl-CoA (Table I) corroborates the notion that Dv3MaT is a "malonyltransferase." The malonyl acceptor specificity of Dv3MaT was highly restricted to the 3-*O*-glucosides of anthocyanidins. The 3-*O*-glucoside of flavonol (quercetin) and 3,5-*O*-diglucosides of anthocyanidins were poor substrates for the enzyme (Table I). The enzyme could efficiently catalyze the malonylation of both the pelargonidin- and cyanidin-types of anthocyanins (see also Fig. 1A), and both types of anthocyanins are found to be malonylated in the flowers. The other enzymatic profiles, including molecular mass (51 kD), optimum pH for catalytic activity (pH 8.5), and strong inhibition by heavy metal ions such as Cu^{2+}, were also consistent with those reported for the native 3MaT activity of dahlia (Yamaguchi et al., 1999). Moreover, transgenic petunia harboring *Dv3MaT* cDNA specifically accumulated monomalonylated cyanidin 3-*O*-glucoside. These results allowed us to conclude that *Dv3MaT* codes for 3MaT participating in the biosynthesis of malonylated anthocyanins in the dahlia flowers and should prove the validity of our approach for the efficient identification of the anthocyanin acyltransferase gene expressed in the flower.

Sequence Comparison Studies

The specificities of anthocyanin acyltransferases are mainly characterized by acyl donor specificity (aromatic acyl-CoA-specific versus aliphatic acyl-CoA-specific) and the regiospecificity of acyl transfer (3-glucoside-specific versus 5-glucoside-specific, etc.). However, the characteristics of the primary structure responsible for these specificities are yet to be identified. So far, four sequences of anthocyanin acyltransferases with established specificities have been published: Gt5AT (a 5-glucoside-specific aromatic acyltransferase of *G. triflora*), Pf3AT (a 3-glucoside-specific aromatic acyltransferase of *P. frutescens*), and Ss5MaT1 and Pf5MaT (5-glucoside-specific aliphatic acyltransferases of *S. splendens* and *P. frutescens*, respectively). Because the Dv3MaT obtained in this study was the first example of a 3-glucoside-specific aliphatic acyltransferase, the enzymes of four possible combinations of specificities are now available from four different plant species, and this allows us to explore the primary structural characteristics that may be responsible for these specificities.

The alignment of these available sequences (Fig. 2) shows several conservations of amino acid residues within each set of sequences of enzymes exhibiting the same acyl donor specificity. Arg-126 of Dv3MaT, particularly, is found to be invariant in the sequences of all malonyltransferases compared here (Fig. 2). Also, basic amino acid residues (Lys and Arg) are conserved at a position corresponding to position 365 of Dv3MaT. It is likely that one or both of these cationic residues are involved in the specific binding of malonyl-CoA via electrostatic interactions in these malonyltransferases, and this warrants future site-directed mutagenesis studies of Dv3MaT to probe the role of Arg-126 and Lys-365 in the malonyl-CoA binding. Some aromatic amino acid residues similarly are found conserved in the sequences of Gt5AT and Pf3AT (e.g. Tyr-98, Phe-182, Tyr-314, and Phe-396 of Gt5AT), one of which may be responsible for possible aromatic interactions in the specific binding of aromatic acyl-CoA by these aromatic acyltransferases. In this case, however, a comparison among larger numbers of sequences of aromatic acyltransferases from different plant species will be necessary to further identify the residue(s) responsible for the binding of aromatic acyl-CoA. In addition, it was difficult to find any relevance of the characteristics of the amino acid sequence or residue(s) to the regiospecificity of acyl transfer.

Functional Significance of Anthocyanin Malonylation

To examine the effect of anthocyanin malonylation on the flower color, we created transgenic petunia plants harboring *Dv3MaT* cDNA and analyzed their anthocyanin compositions and flower colors. The 13 transgenic lines of petunia were found to accumulate *Dv3MaT* mRNA and produce malonylated anthocyanins. The flower colors of these transgenic lines were not significantly different from those of the host, indicating that monomalonylation of anthocyanins (cyanidin 3-*O*-glucoside and cyanidin 3, 5-*O*-diglucoside) is not intrinsically related to flower color. This is consistent with the fact that the absorption spectra of the malonylated anthocyanins were essentially identical with those of the corresponding non-malonylated forms.

We then examined the effect of 6″-*O*-malonylation of anthocyanin on the pigment stability in in vitro aqueous systems at pH 5 to 7, which correspond to the pHs of the intracellular milieus (cytosol and vacuoles) of flowers. The results showed that malonylation greatly enhanced the stability of the color intensities and pH-dependent structural reversibility of anthocyanins in aqueous systems. In addition, malonylation also prevented anthocyanins from the attack of β-glucosidase. Thus, malonylation of anthocyanin should enhance the pigment stability both physicochemically and biochemically to play a very important role in maintaining the color of anthocyanins in the intracellular milieus of flowers. Although mechanistic details of the observed physicochemical stabilization of color of anthocyanins by malonylation remain to be clarified, it is likely that the malonyl group helps to maintain the equilibrium between the flavylium cation and quinonoidal base species of the pigment through the ionization of its free carboxyl group (Saito et al., 1988). In addition, the malonyl group may also make contributions to pigment stability through its involvement in the interactions of the pigment with other vacuolar components such as phenolic compounds (i.e. copigmentation [Yabuya et al., 2000]) and metal ions (Brouillard and Dangles, 1994), as suggested from the observation that color intensity of cyanidin 3-*O*-6″-*O*-malonylglucoside in petal extracts (pH 5.1) of dahlia was highly stable (see "Results").

The results obtained in this study warrant the significance of anthocyanin malonylation in controlling floral colors through genetic engineering approaches. The anthocyanin malonyltransferase gene should be useful in the stabilization of anthocyanin pigments, which enable flower color modification in transgenic plants, although the petunia obtained in this study did not have altered flower color. It could also be mentioned that anthocyanin malonyltransferase genes would be required for the metabolic engineering of pathways leading to polyacylated anthocyanins, in which a series of enzymatic modifications of anthocyanins may proceed in a sequential manner, and that the malonylation of anthocyanins should provide the necessary intermediates for subsequent modifications. Overexpression of malonyltransferase genes in heterologous plants may compete out endogenous anthocyanin modification and result in color modified plants.

MATERIALS AND METHODS

Plant Materials and Anthocyanins

Dahlia (*Dahlia variabilis* cv Kaen) plants were grown at the farm of Minami-Kyushu University and separated into leaves, stems, bracts, sepals, and petals of three developmental stages (see legend to Fig. 3). Also, entirely white petals (15–20 mm in length) were separated from fully open flowers of the acyanic (white) cultivar of dahlia grown at the farm of Tohoku University (Miyagi, Japan). The isolated organs were stored at −80°C until use. An in-house red variety of petunia (*Petunia hybrida*) that accumulates cyanidin 3-*O*-glucoside and 3,5-*O*-diglucoside was grown in a greenhouse. Anthocyanins were isolated and purified from petals as described previously (Yamaguchi et al., 1999; Suzuki et al., 2001) and used as authentic samples after confirmation of their structures by instrumental analyses. Quercetin 3-*O*-glucoside was kindly provided by Prof. T. Iwasina (Tsukuba Botanical Garden, National Science Museum, Tsukuba, Japan).

cDNA Cloning

Poly(A^+) RNA from the buds of dahlia was used for the construction of a cDNA library using the λZAPII-cDNA synthesis kit (Stratagene, Heidelberg). A reverse primer, 5′-TTICCCCAICC(G/A) AA(G/A) TC-3′, was designed based on the amino acid sequence Asp-Phe-Gly-Trp-Gly (motif 3; see Suzuki et al., 2001). A forward primer, 5′-AT(T/C) TA(T/C) TT(T/C) GGIAA(T/C) TG-3′, was based on the sequence Asn-Tyr-Phe-Gly-Asn-Cys (motif 2). The bases, which are in parentheses, indicate the degenerate sites. The cDNA library was then used as a template for PCR amplification using the above-mentioned PCR primers. The amplified fragment, *AT927*, was cloned into TOPO-pCR2.1 (Invitrogen, Carlsbad, CA) and subjected to sequencing using a Dye-Terminator Cycle Sequencing Kit (Beckman Coulter, Fullerton, CA) with a CEQ 2000 DNA analysis system (Beckman Coulter).

The *AT927* fragment was DIG-labeled using the PCR DIG Probe Synthesis Kit (Roche Diagnostics, Basel). The cDNA library was screened by plaque hybridization with the amplified cDNA fragment as a probe using the PCR DIG Probe Synthesis Kit and the DIG-DNA Labeling and Detection Kit (Roche Diagnostics). The hybridization was performed at 37°C in 5× SSC containing 0.02% (w/v) SDS, 0.1% (w/v) *N*-lauroylsarcosine, 2% (w/v) blocking reagent, and 30% (v/v) formamide (Roche Diagnostics). The filters were washed two times in 0.1× SSC and 0.1% (w/v) SDS at 55°C for 15 min.

Quantitative Real-Time PCR

Total RNA was prepared from the individual organs of dahlia plant using the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany). The quality of the RNA was confirmed by visualization by UV-light irradiation of ethidium bromide-stained rRNA separated on 2.5% (w/v) agarose gel containing 2% (v/v) formaldehyde. First-strand cDNA was synthesized with an oligo$(dT)_{12–18}$ primer and reverse transcriptase, SuperScript II (Invitrogen), from 1 μg of total RNA. The *Dv3MaT* transcript was quantified by quantitative real-time PCR on the LightCycler Quick System model 330 (Roche Diagnostics), where two *Dv3MaT*-specific primers (5′-CGATTACGTCACACTCCCGGTTTTCTCG-3′ and 5′-CGTCTTCACCTTTCTTTTCCCCCATGTTGAC-3′) and the first-strand cDNA were used as a template along with the LightCycler-FastStart DNA Master SYBR Green I Kit (Roche Diagnostics). Thermal cycling conditions were 95°C for 2 min followed by 45 cycles of 95°C heating pulse for denaturation and 72°C for 41 s for annealing and extension. The amplified DNA fragment (450 bp) was sequenced to confirm that the amplified fragment codes for a partial *Dv3MaT* cDNA.

Heterologous Expression of Dv3MaT in *Escherichia coli*

To introduce an *Sac*I site and a *Kpn*I site, PCR was performed using a full-length *Dv3MaT* cDNA (cloned into the plasmid pBluescript SK$^-$) as a template and primers 5′-GAGCTCATGGACAACATTCCC-3′ and 5′-GGTACCTTACGACGCAATTGC-3′, respectively. The amplified fragment was digested with *Sac*I and *Kpn*I and then ligated with a pQE-30 vector (Qiagen), yielding pDv3MaT, which was subsequently used to transform *E. coli* JM109 cells. Dv3MaT was expressed in the transformant cells and purified to apparent homogeneity, essentially as described previously (Suzuki et al., 2001).

Enzyme Assays

The standard reaction mixture (100 μL) consisted of 20 mM potassium phosphate, pH 7.0, 120 μM pelargonidin 3-*O*-glucoside, 60 μM malonyl-CoA, and enzyme. After incubation at 30°C for 20 min, the reaction was stopped by adding 200 μL of ice-cold 0.5% (v/v) trifluoroacetic acid. Anthocyanins in the resultant mixture were analyzed by reversed-phase HPLC using the Dynamax system (Rainin Instruments Co., Woburn, MA) equipped with an SPD-10A *VP* UV-VIS detector (Shimadzu, Kyoto) with detection at 510 nm; column, Asahipak ODP-50 4E (4.6 × 250 mm; Shodex, Shoko, Tokyo); and flow rate, 0.7 mL min^{-1}. For the analysis of monoglucosylated anthocyanins, the following conditions (system A) were used. The analytical column was previously equilibrated with 18% (v/v) acetonitrile in 0.5% (v/v) trifluoroacetic acid in water. After injection, the column was initially developed isocratically with 18% (v/v) acetonitrile for 3 min, followed by linear gradients from 18% to 28% in 15 min and from 28% to 50% in 1 min. The column was then washed isocratically with 50% (v/v) acetonitrile for 4 min, followed by a linear gradient from 50% to 18% in 1 min. Diglucosylated anthocyanins were analyzed under the following conditions (system B). The analytical column was previously equilibrated with 15% (v/v) acetonitrile in water. After injection, the column was initially developed isocratically with 15% (v/v) acetonitrile for 3 min, followed by linear gradients from 15% to 28% in 15 min and from 28% to 50% in 1 min. The column was then washed isocratically with 50% (v/v) acetonitrile for 4 min, followed by a linear gradient from 50% to 15% in 1 min.

Construction of Binary Vectors and Petunia Transformation

The binary vector, pBE2113-GUS, where the β-glucuronidase gene is between the enhanced cauliflower mosaic virus 35S promoter and the *nos* terminator (Mitsuhara et al., 1996), was digested with *Sac*I, blunted, and ligated with a *Sal*I linker to yield pBE2113-GUSs. The cDNA of *Dv3MaT* was digested with *Nco*I, blunted, and then digested with *Xho*I. The resultant 1.6-kb fragment was ligated with the vector of *Sna*BI-*Sal*I digested with pBE2113-GUSs to yield pSPB1324. The red petunia was transformed as described by Horsh et al. (1985) using *Agrobacterium tumefaciens* strain AGL0 (Lazo et al., 1991).

Analysis of Transgenic Petunia Plants

Total RNA was prepared from the petals of open flowers using the RNeasy Plant Mini Kit (Qiagen). One microgram of the total RNA was subjected to RT-PCR analysis using the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen) and a pair of primers (5′-CCCGTCCATCATCTTTACTTCTACC-3′ and 5′-CACCATTTGCTTATCC-CCTCTC-3′) and then analyzed with agarose gel electrophoresis.

Weighed quantities of petals (1 g; calyces were removed) of transgenic (T1) and non-transgenic petunia were pulverized in 2 mL of 0.1% (v/v) trifluoroacetic acid in methanol in a mortar. The extracts were centrifuged at 1,000*g* for 10 min. The supernatant was filtered through a 0.45-μm filter and analyzed for anthocyanins by an HPLC-MS system equipped on-line with a photodiode-array spectroscopic detector (model G1315A, Agilent Technologies, Palo Alto, CA; 250–600 nm) and a mass spectrometer (LC-Q MS detector, Thermo Finnigan, San Jose, CA) with electrospray ionization by positive mode. HPLC (system C) was conducted using a Shodex DE-413L column (4.6 mm x 250 mm; Shoko Co., Ltd., Japan) at a flow rate of 0.5 mL min^{-1}. After injection of the sample, the column was developed with a linear gradient in 10 min from 10% to 50% (v/v) acetonitrile containing 0.5% (w/v) trifluoroacetic acid in water, followed by an isocratic elution for 15 min of acetonitrile containing 0.5% (w/v) trifluoroacetic acid in water. Genomic DNAs (20 μg) of transgenic and non-transgenic lines of petunia were prepared from their leaves, digested with *Hin*dIII, and subjected to Southern-blot analysis with the *Dv3MaT* cDNA as a probe using the DIG DNA Labeling and Detection Kit (Roche Diagnostics).

Stability of Color Intensity of Cyanidin 3-*O*-6′-*O*-Malonylglucoside in Petal Extract

Petals (1 g; calyces were removed) of dahlia cv Kaen were pulverized in 4 mL of Milli-Q grade water containing 5 mM 2-mercaptoethanol in a mortar. The extract was centrifuged at 10,000*g* for 30 min. An appropriate amount of cyanidin 3-*O*-6″-*O*-malonylglucoside was added to the extract, whose pH was 5.1, and the mixture was incubated at 30°C for 1 h. A_{520} of the extract was measured before and after the incubation using a double-beam spectrophotometer (model U-2000, Hitachi), and the percentages of initial absorbance after the 1-h incubation were determined.

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